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Two homologous oligopeptide binding protein genes (*oppA*) in *Lactococcus lactis* MG1363

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Abstract

In previous studies, it has been shown that inactivation of *opp* or even *oppA* abolishes the capacity of *Lactococcus lactis* to utilize oligopeptides. We now show that the *opp* operon has been duplicated in *L. lactis* MG1363. The nucleotide sequence of the *oppA* and *oppC* homologues (*appA* and *appC*) and most of the *oppB* homologue (*appB*) indicate that the corresponding protein sequences are 83%, 92% and 91% identical, respectively. Inactivation of *appA*, via homologous recombination, as well as complementation studies were carried out to determine the possible function of *appA* in peptide utilization. As anticipated from studies with an *oppA* knock-out, peptide utilization was not impaired in an *appA* disruption mutant. Importantly, AppA expressed from a plasmid could restore the ability of *oppA* deletion mutants to utilize Leu-enkephalin, albeit with a lower efficiency than OppA. The differences in the ability to utilize this pentapeptide were not due to differences in expression levels but most likely reflect a different catalytic efficiency in oligopeptide utilization when AppA is used as ligand receptor.

Keywords: *Lactococcus lactis*; Oligopeptide transport system; Gene duplication; Gene complementation

1. Introduction

The proteolytic pathway for the utilization of milk proteins as exogenous nitrogen source is well-described in *Lactococcus lactis*. The system is composed of an extracellular proteinase, at least three peptide transport systems, one specific for oligopeptides (Opp) and two others for di- and tripeptides (DtpT and Dpp), and a set of intracellular peptidases (Kunji et al., 1996b; Sanz et al., 2001). The extracellular proteinase degrades caseins predominantly into oligopeptides of 5–26 residues, which are exclusively substrates of Opp. The intracellular peptidases contribute to the complete degradation of oligopeptides into free amino acids. Thus, Opp is considered essential for nutrition, while the di-tripeptide transport

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systems seem to have a role in regulation (Marugg et al., 1995; Kunji et al., 1996a; Meijer et al., 1996; Sanz et al., 2001). The Opp system belongs to the ABC-transporter superfamily and consists of a peptide binding protein (OppA), two integral membrane proteins (OppB and OppC) and two ATP-binding proteins (OppD and OppF; Tynkkynen et al., 1993). In general, the solute binding proteins provide the primary interaction site for the ligand and largely define the specificity of the transport system (Sleigh et al., 1997). Some binding-protein dependent ABC-transporters, however, function with more than one ligand binding protein, each with a different specificity (Ehrmann et al., 1998). In Gram-positive bacteria, this is exemplified by the three-homologous binding proteins working together in the Ami system of *Streptococcus pneumoniae* (Alloing et al., 1994). In *L. lactis*, the transport of oligopeptides has been exclusively attributed to the Opp system and the binding protein OppA is considered essential for its function.

Here, we report on the identification and characterization of the genes specifying a putative second oligopeptide transport system in *L. lactis* MG1363. Complete sequences of the *oppA* and *oppC* counterparts (*appA* and *appC*) and part of the *oppB* counterpart (*appB*) are presented. The possible functionality of *appA* in peptide utilization has been determined in a gene knockout and by complementation studies.

2. Materials and methods

2.1. Strains and culture conditions

Escherichia coli strains were grown, at 37 °C, in Luria-Bertani broth (Sambrook et al., 1989). Ampicillin or erythromycin was added to 100 μg ml^{-1}, when appropriate. *L. lactis* strains were routinely grown, at 30 °C, in M17 broth (Difco, East Molesey, UK) supplemented with 0.5% (w/v) glucose. The ability of the *L. lactis* strains to utilize peptides was determined in CDM (Poolman and Konings, 1988) containing all amino acids except an essential one that was supplied in the form of a di-, tri- or oligopeptide, as described elsewhere (Kunji et al., 1993). Erythromycin was added to 5 μg ml^{-1}, when appropriate.

2.2. General DNA manipulation and sequence analysis

Standard molecular techniques were essentially performed as described by Sambrook et al. (1989). Chromosomal and plasmid DNA were isolated by the methods of Birnboim and Doly (1979) and Leenhouts et al. (1990). Southern analysis was carried out by the non-radioactive DNA Labeling and Detection kit (Boehringer, Almere, The Netherlands). *E. coli* and *L. lactis* were transformed by electroporation as described by Zabarovsky and Winberg (1990) and Holo and Nes (1989), respectively. Sequence analyses were carried out by the dideoxy-chain termination method (Sanger et al., 1977). Sequence similarity searches were done with the FASTA algorithm of Pearson and Lipman (1988). Sequence alignments were performed with DNAman program (Version 4.03).

2.3. Plasmid construction

The *appA* gene was inactivated in the double peptide transport mutant *L. lactis* AG500 (Δ*opp*, Δ*dtpT*, Hagting et al., 1994; Kunji et al., 1996b) by plasmid integration via homologous recombination (Leenhouts et al., 1989). A 1.25-kb fragment of the *oppA* homologue (*appA*) was initially detected in a genome library of *L. lactis* MG1363 in *E. coli* NM522 (Buist et al., 1995). The 1.25-kb *appA* fragment in p15D4 (Ap^r, pUC19 derivative) was inserted at the *Hind*III/*Asp*718 sites of the integrative vector pORI28 (Em^r) (Leenhouts and Venema, 1993). *L. lactis* AG500 was transformed with the new construct, p15D14dis, to achieve the inactivation of *appA*, which was verified by Southern hybridization. The expression vector of *appA* was constructed as follows. The gene *appA* was amplified from chromosomal DNA of *L. lactis* AG500 and the PCR product was inserted at *Nco*I/*Bam*HI sites of pAMP31 (Picón et al., 2000), thereby replacing *oppA* for *appA*. In this vector, *appA* is under the control of the constitutive lactococcal promoter *P32* (Van der Vossen et al., 1987) and fused to a 3′ -end sequence specifying a six-histidine tag. The resulting vector, pYSAprime, was transformed into the *oppA* deletion mutant *L. lactis* AMP2 (Picón et al., 2000).

2.4. Western analysis

The expression of *oppA* and *appA* from the corresponding plasmids in whole-cell lysates of *L. lactis* AMP2 was quantified by Western analysis using monoclonal antibodies (Dianova, Apeldoorn, The Netherlands) raised against the 6-histidine tag (Sanz et al., 2000).

2.5. Nucleotide sequence accession number

The sequence data have been submitted to the GenBank database under accession number AF245305.

3. Results and discussion

3.1. Identification of opp homologues and sequence analysis

In the chromosome of *L. lactis* MG1363 the existence of a DNA fragment homologous to *oppA* was revealed by Southern analysis (data not shown). Subsequently, a gene library of *L. lactis* MG1363 in *E. coli* NM522 (Buist et al., 1995) was screened for hybridization with a probe consisting of a 750-bp *oppA* fragment. Plasmid DNA from positive colonies was isolated and checked by Southern analysis. The insert of 1.25 kb in the smallest plasmid containing part of this *oppA* homologue (p15D4) was sequenced, which revealed the presence of an incomplete open reading frame (ORF3) that was 81% identical to *oppA* at the nucleotide level and, therefore, designated *appA* (Fig. 1). The 5′-end (56 codons), an extra fragment towards the 3′-end (80 codons) and the region upstream of *appA* were obtained by inverse PCR (IPCR) on a 2.5-kb *Hind*III chromosomal fragment (Fig. 1). The region (1.8 kb) upstream of *appA* comprises a complete (ORF2) and an incomplete (ORF1) ORF. ORF1 comprises 228 codons and was designed *appB* although 49 codons are missing when DNA is aligned with *oppB*. The deduced protein AppB and OppB are 90.7% identical. ORF2 comprises 294 codons and the deduced protein, AppC, is 91.5% identical to OppC. *appC* is preceded by a putative ribosome-binding site (RBS) with a ΔG° of -12.8 kcal/mol. The intergenic region of *appC* and *appA* is 115 bp long and it contains consensus promoter sequences and a putative RBS with AG° of -9.4 kcal/mol (Van der Vossen et al., 1987). The sequence of the 3′-end of *appA* and the downstream region were obtained by IPCR on a 1.7-kb *Ssp*I chromosomal fragment (Fig. 1). The complete sequence of *appA* comprises 596 codons, which is four codons less that *oppA*, and the corresponding protein sequences are 82.8% identical. The region with the largest dissimilarity is position 235–276 (Fig. 2). Unlike for *oppA*, which is followed by the endopeptidase gene *pepO*, the region downstream of *appA* contains two inverted repeats (ΔG° of -10.5 and -5.7 kcal, respectively) that could function as a rho-independent terminator.

3.2. Inactivation of appA and functional characterization of disruption mutants

The gene *appA* was inactivated by plasmid integration via homologous recombination. One of the *appA*

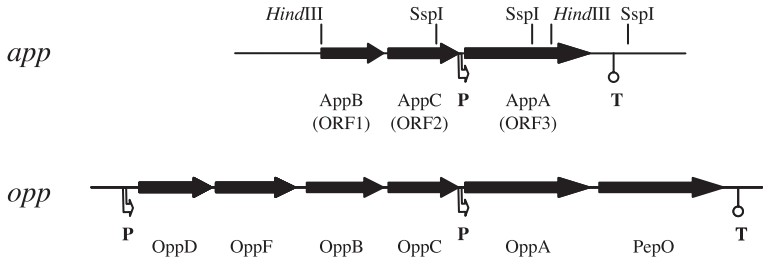

Fig. 1. Schematic representation of the *opp* operon (Tynkkynen et al., 1993) and the sequenced chromosomal DNA region of *L. lactis* MG1363 containing the *opp* homologues, *appA*, *appC* and most of *appB*. The positions of putative promoter regions (P) and rho-independent terminators (T) are indicated. The restriction sites used for inverse PCR amplifications are indicated. The nucleotide sequence data will appear in the GenBank under accession number AF245305.

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AppA        MKKLNITLLATTAVLATALLSACGSNQSSSTSTTKLKAGS     40
OppA        MnKLkvTLLAssvVLAatLLSACGSNQSSSTSTkKLKAGn     40

AppA        FDVAYKNPDKAIKGGDLKVAYQSDSPMKAEWWSGLENDAT     80
OppA        FDVAYqNPDKAIKGGnLKVAYQSDSPMKAqWlSGLsNDAT     80

AppA        FAAMASPAGGLDGIFFTNSAFKFINGGPANISLDDSAKTA    120
OppA        FAtMsgPgGGqDGlFFTdSgFKFIkGGaAdvaLDkesKTA    120

AppA        TITLRKDLKWSDGSQVTAKDYEFTYETIANPAYGSDRWTD    160
OppA        TITLRKDLKWSDGSeVTAKDYEFTYETIANPAYGSDRWTD    160

AppA        SLANIVGLSDYHTGKAKTISGITFPDGENGKVIKIQFKEM    200
OppA        SLANIVGLSDYHTGKAKTISGITFPDGENGKVIKvQFKEM    200

AppA        TPGMTQSGNGYFLETVTPYQYLKDVAPKDLASSPKRQ.QT    239
OppA        kPGMTQSGNGYFLETVaPYQYLKDVAPKDLASSPKtttkp    240

AppA        ISHRSFQTANVVAGD.QSICTNPYYWA.KTKLT.LSPMNS    276
OppA        lvtgpFkpeNVVAGesikyvpNPYYWgeKpKLnsityevv    280

AppA        STNKSVAALSSGKYDFINSMVASQYKQVKNLKGYKVLGQQ    316
OppA        STaKSVAALSSsKYDiINgMVsSQYKQVKNLKGYKVLGQQ    320

AppA        ALYISLMYYNLGHYDTKNSINVQDRKTPLQDQSVRQAVAY    356
OppA        AmYISLMYYNLGHYDaKNSINVQDRKTPLQDQnVRQAigY    360

AppA        ARNVAEVDNKFSNGLATPANGLIPPIFKQFTSSSVKGYEK    396
OppA        ARNVAEVDNKFSNGLsTPANsLIPPIFKQFTSSSVKGYEK    400

AppA        QNLDKANKLLDADGWKLNKSTGYREKDGKELSLVYAARQG    436
OppA        QdLDKANKLLDeDGWKLNKSTGYREKDGKELSLVYAARvG    440

AppA        DANQETIAQNYIQQWKKIGVKVSLYNGKLMEFNSWVDHMT    476
OppA        DANaETIAQNYIQQWKKIGVKVSLYNGKLMEFNSWVDHMT    480

AppA        TPPGADDWDITDGAWSLSSEPSQQDLFSAAAPYNIGHFND    516
OppA        TPPGAnDWDITDGsWSLaSEPSQQDLFSAAAPYNfGHFND    520

AppA        PQITNDLNDIDSTKSESATYRKAAFVKYQNDMNKKAYVVP    556
OppA        seITkDLNDIDSaKSEnpTYRKAAFVKYQeDMNKKAYViP    560

AppA        T.FHLN.PPVNKRLVGMTLDYGDMNLWSEIGVSSNKMATK    594
OppA        TnFmLNytPVNKRvVGMTLDYGaMNtWSEIGVSSaKlATK    600
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Fig. 2. Comparison of OppA and AppA proteins of *L. lactis* MG1363. The sequences were aligned with the DNAman program (Version 4.03). Identical residues in both sequences are in capital letters.

disruption mutants, designated *L. lactis* YS32, was selected and its phenotype was analyzed. It has been shown that the Opp system is responsible for the uptake of oligopeptides (>3 residues), and that the binding protein, OppA, is an essential component of this function (Kunji et al., 1996a). On the other hand, the existence of residual di- and tripeptide uptake in triple mutants, lacking functional Opp, DtpT and Dpp, suggests the presence of a fourth distinct uptake system (Sanz et al., 2001). Following these observations, the *appA* disruption mutant was tested for its ability to utilize di- and tripeptides. Growth experiments were carried out in CDM containing all amino acids except an essential one that was supplied in the form of a di- or tripeptide (Poolman and Konings, 1988). Growth rates on several peptides of *L. lactis* YS32 and AG500, carrying the empty vector pAMP0 (Picón et al., 2000) were not significantly different (Fig. 3). Thus, *appA* does not seem to have a role in di- and tripeptide utilization.

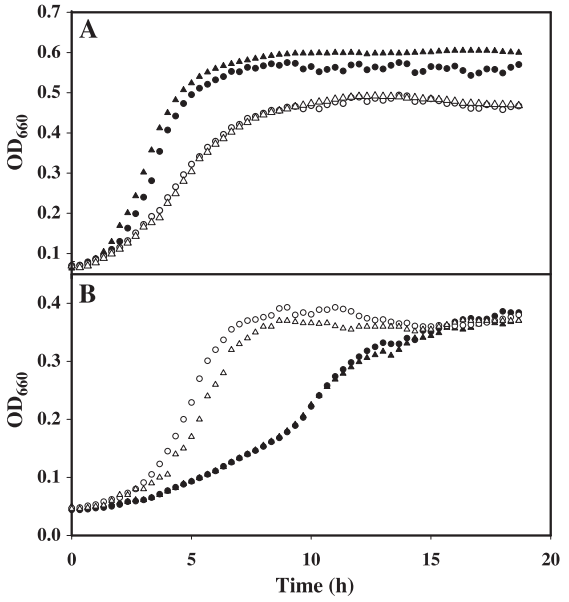

Fig. 3. Growth of *L. lactis* strains in CDM containing a di- or tripeptide as the sole source of the essential amino acids. (A) CDM-Leu + 0.09 mM Leu-Leu: (●) AG500 (pAMP0) and (▲)YS32 (*appA*::p15D14dis); CDM-Leu + 0.18 mM Leu-Gly-Gly: (O) AG500 (pAMP0) and (Δ)YS32 (*appA*::p15D14dis); (B) CDM-Val + 0.28 mM Val-Val: (●) AG500 (pAMP0) and (▲)YS32 (*appA*::p15D14dis); CDM-Val + 0.58 mM Val-Gly-Gly: (O) AG500 (pAMP0) and (Δ)YS32 (*appA*::p15D14dis).

3.3. Expression of appA from a plasmid and role of appA in oligopeptide utilization

To determine whether or not *appA* encodes a functional binding protein, the gene was placed under the control of the constitutive lactococcal promoter *P32*. The expression of *oppA* and *appA* from the corresponding plasmids in *L. lactis* AMP2 (Δ*oppA*) was quantified by Western analysis. Both proteins were expressed to similar levels (data not shown). Since the utilization of the pentapeptide Leu-enkephalin is characteristic for Opp activity, *L. lactis* AMP2 (pYSAprime) was tested for its ability to grow in CDM with Leu-enkephalin as the sole source of leucine. Growth rates of wild-type (MG1363) and the *oppA* deletion mutant AMP2, carrying either pAMP31 or pYSAprime, on different concentrations of Leu-enkephalin (0.18, 0.09 and 0.03 mM) were determined. As anticipated, the deletion mutant, *L. lactis* AMP2, was unable to grow on any of these concentrations of Leu-enkephalin as the only source of leucine and each strain grew with similar rates when leucine was present (data not shown). The *appA* in pYSAprime complemented *oppA* with respect to Leu-enkephalin utilization, but the growth rate was much lower (Fig. 4). Growth rates of the wild-type (MG1363, data not shown) and the strain AMP2 (pAMP31) on Leu-enkephalin were similar ($\mu \sim 0.60\ h^{-1}$ at 0.18 mM Leu-enkephalin and $\sim 0.53\ h^{-1}$ at 0.09 mM Leu-enkephalin). Growth rates of AMP2 (pYSAprime) were considerably lower ($0.21\ h^{-1}$ at 0.18 mM Leu-enkephalin and $0.14\ h^{-1}$ at 0.09 mM Leu-enkephalin), and growth was almost impaired at lower concentrations (0.03 mM) while MG1363 and AMP2 (pAMP31)

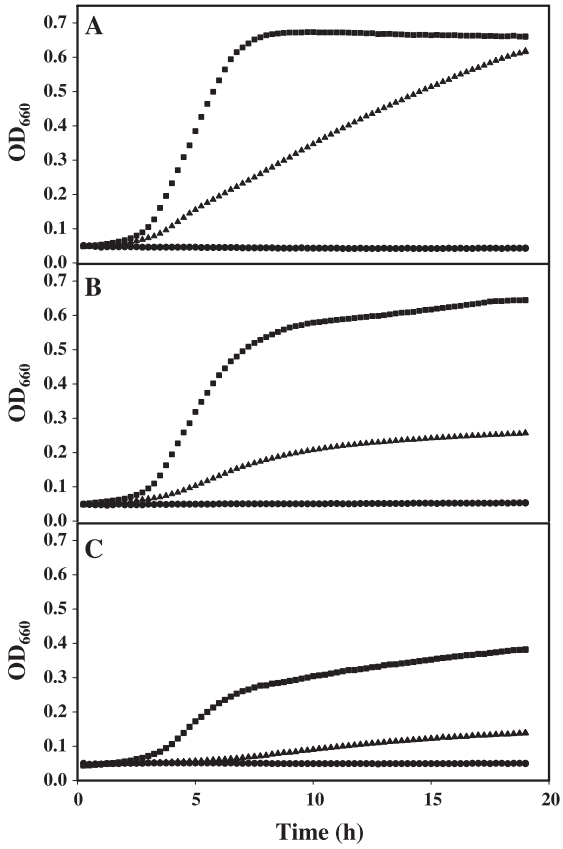

Fig. 4. Growth of *L. lactis* strains in CDM containing Leu-enkephalin as the sole source of the essential amino acid leucine. (A) 0.18 mM Leu-enkephalin; (B) 0.09 mM Leu-enkephalin; (C) 0.03 mM Leu-enkephalin. *L. lactis* strains: (●) AMP2; (■), AMP2 (pAMP31); (▲), AMP2 (pYSAprime).

grew to some extent. As these effects cannot be attributed to differences in expression of OppA and AppA, they most likely reflect the inefficient donation of the peptide by AppA to the translocator complex.

The absence of growth of *oppA* deletion mutants on Leu-enkephalin, and the complementation of *oppA* by *appA* when provided in trans suggest that the chromosomal copy of *appA* is not functional or poorly expressed. Western analysis of cell lysates of *L. lactis* AG500 confirms this notion using antibodies raised against OppA which also reacts to AppA (data not shown). Nevertheless, important structure–function relationships might be obtained from the dissimilarities between both proteins. The three dimensional structure of the peptide binding protein OppA of *Salmonella typhimurium* in complex with different ligands has made it possible (Sleigh et al., 1997; Tame et al., 1994, 1995). A first class of OppA specificity mutants has been constructed in *L. lactis* on the basis of multiple alignments with OppA form *S. typhimurium* (Picón et al., 2000). Future progress in defining residues of OppA critical for ligand binding can be made on the basis of sequence comparisons with the newly identified AppA.

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